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Biochemical and Electrophoretic Studies of Erythrocyte Pyridoxine Kinase in White and Black Americans

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Pyridoxine kinase (PNK; EC 2.7.1.35) catalyzes the phosphorylation of pyridoxine to its active coenzyme form, pyridoxine phosphate:

$$\text{pyridoxine} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{PNK}} \text{pyridoxine phosphate} + \text{ADP}.$$

The same enzyme catalyzes the phosphorylation of pyridoxal to pyridoxal phosphate.

Previous communications [1, 2] have shown that the average PNK activity in red cells of American blacks is significantly lower than that of American whites; in contrast, the activities of the enzyme in lymphocytes, granulocytes, and cultured skin fibroblasts from black subjects are the same as those from whites.

To gain a better understanding of the genetics of the red cell PNK of blacks, the size of our population survey has been expanded, and some limited family studies have been carried out. To determine whether the enzyme deficiency of black subjects is due to a structural gene mutation or regulatory mutation, we have investigated further the biochemical characteristics of PNK in red cells of white and black donors. The results of our investigations suggest that low red cell PNK activity may be coded by a single structural allele which results in the production of an enzyme with reduced in vivo stability.

MATERIALS AND METHODS

Pyridoxine kinase activity was estimated by measuring ^{3}H-pyridoxine-5′-phosphate as described previously [3]. Assay results were found to be highly reproducible in the same subject at different times. The red cells of one normal white subject were assayed four times over a 2 month period. A mean enzyme activity of 1.461 mU/g Hb with a standard deviation of 0.087 mU/g Hb were found. In the case of a subject with low activity, three determinations over a span of 1 month gave a PNK activity averaging 0.474 mU/g Hb with a standard deviation of 0.029 mU/g Hb. Partially purified enzyme solutions from red cells of white and black subjects (white donor: 1.496 mU/g; black donor: 0.509 mU/g Hb; white subject with enzyme deficiency: 0.495 mU/g Hb) were prepared by

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DEAE-cellulose chromatography followed by ammonium sulfate precipitation with 55% saturation [4]. The enzyme suspension was centrifuged; the precipitate was dissolved in 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol and dialyzed against the same buffer before use. Density separation of red cells was carried out by the procedure of Murphy [5]. Samples were taken from the most dense, the least dense, and the middle portion of the cells. Hexokinase activity was measured as an indicator of red cell age [6]. Comparison of biochemical properties of PNK from black and white subjects was always conducted concurrently under identical experimental conditions.

Electrophoresis with 10.4% Electrostarch (Otto Hiller, Madison, Wisconsin) was performed at pH 7.0 in the vertical system described by Smithies [7]. A 0.1 M potassium phosphate buffer, pH 7.0, was used in the electrode compartments and 10 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol was employed as gel buffer. Approximately 40 μl of the dialyzed PNK solution from either a white donor (9.49 mU/ml), a black donor (4.53 mU/ml), or a 1:1 mixture of the two were placed in each slot. After electrophoresis for 17 hr at 150 V (across the electrodes) and at 20 mA/gel, the gel was sliced and overlaid with a piece of filter paper which was saturated with the following staining mixture: 0.1 M Tris-HCl, pH 8.0; 0.1 M KCl; 0.01 M $MgCl_2$; 0.2 mM NADH; 0.5 mM ATP; lactate dehydrogenase (7.8 U/ml); pyruvate kinase (10.2 U/ml); 5 mM phosphoenolpyruvate; and 0.2 mM pyridoxine. The gel was stained at 37°C for 2–3 hr. At the location of PNK activity on the gel, defluorescence occurs because of the following three reactions:

$$(1)\ \text{pyridoxine} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{PNK}} \text{pyridoxine-5'-phosphate} + \text{ADP};$$

$$(2)\ \text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{Mg}^{++},\ \text{K}^{+}]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate; and}$$

$$(3)\ \text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}.$$

In the absence of ATP, no band of activity is detected; in the absence of pyridoxine, faint bands of activity are found. It is probably due to the presence in the PNK preparation of unknown phosphatases or kinases which utilize ATP. Therefore, it is necessary to perform a control (without pyridoxine) stain of one side of each gel.

RESULTS

Population Studies

The distribution of red cell PNK activity in 51 unrelated white and 52 unrelated black individuals is shown in figure 1. These data include those obtained from 25 black and 36 white subjects reported previously [2]. Using Student's t test and Snedecor's F test (variance ratio test), the data from the larger population does not differ significantly from the smaller population originally reported. The PNK activity of the white donors was 1.305 $\pm$ 0.329 mU/g Hb (mean $\pm$ 1 SD) while that of the black donors was 0.754 $\pm$ 0.305 mU/g Hb. On the basis of the inflections in a probability plot of these data (fig. 2), three ranges of enzyme activity were provisionally defined: PNK^L (low) $<$ 0.625 mU/g Hb, PNK^I (intermediate) 0.626–1.20 mU/g Hb, and PNK^H (high) $>$ 1.2 mU/g Hb. Estimates of the gene frequencies for the two alleles, PNK^H (the common allele in whites which codes for higher enzyme activity) and PNK^L (the common allele in blacks which codes

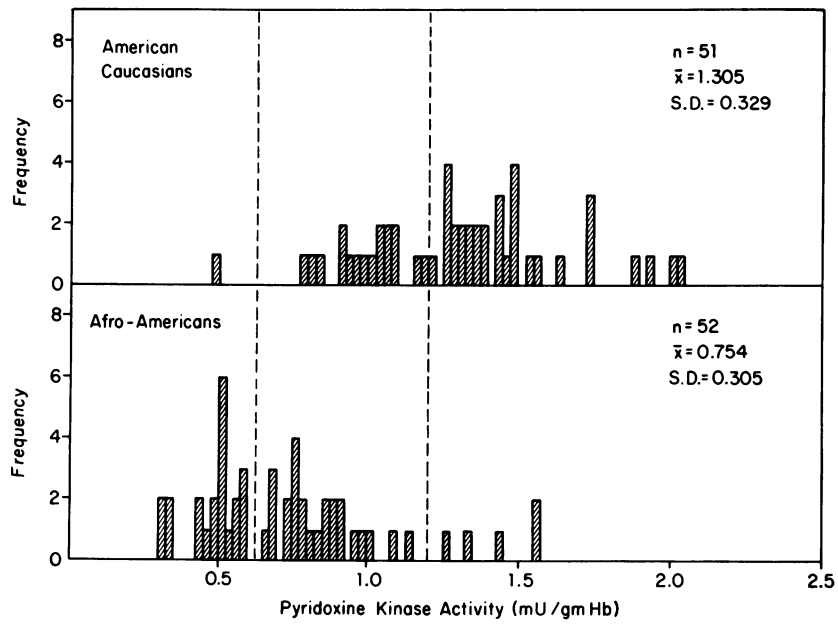

FIG. 1.—Distribution of individual PNK activity in red blood cells from American whites and blacks. Frequency is expressed as no. of individuals. Dashed lines are boundaries for defined homozygous, heterozygous, and normal states of enzyme activity.

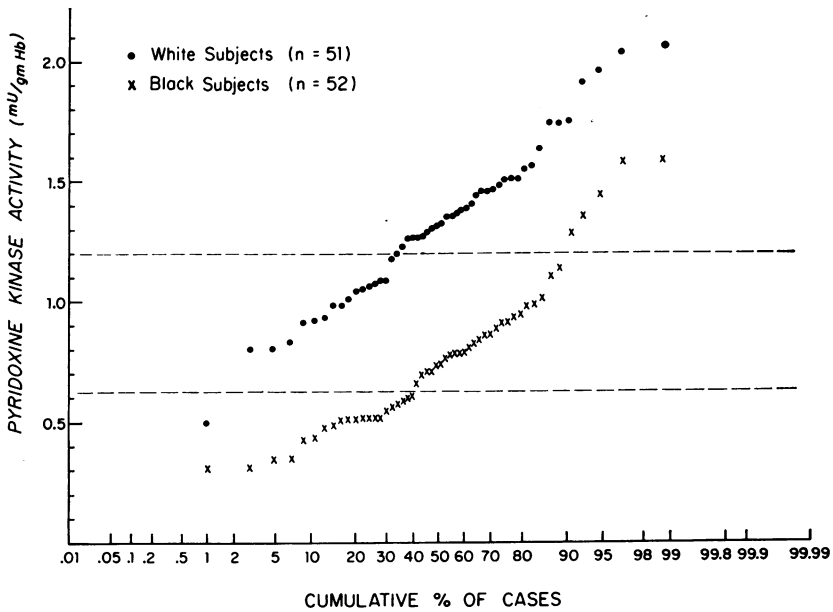

FIG. 2.—Plotting of probability against individual PNK activity in red cells for 51 whites and 52 blacks. Dashed lines are boundaries for defined homozygous, heterozygous, and normal states of enzyme activity.

for lower enzyme activity) may be made assuming that individuals with the PNK^L phenotype are PNK^L/PNK^L, PNK^I phenotype PNK^H/PNK^L, and PNK^H phenotype PNK^H/PNK^H. Calculations from the population data give as $PNK^H = .35$ and $PNK^L = .65$ in the black population and $PNK^H = .81$ and $PNK^L = .19$ in the white population. Table 1 shows good agreement between the number of each

TABLE 1

DISTRIBUTION OF PYRIDOXINE KINASE PHENOTYPE

POPULATION	PHENOTYPES		
	PNK^H	PNK^I	PNK^L
(Presumed Genotypes)	PNK^H/PNK^H	PNK^H/PNK^L	PNK^L/PNK^L
Blacks:			
Observed	5.00	26.00	21.00
Expected	6.37	23.66	21.97
Whites:			
Observed	33.00	17.00	1.00
Expected	33.46	15.70	1.84

NOTE.—Estimated gene frequencies: blacks, $PNK^H = .35$, $PNK^L = .65$; whites, $PNK^H = .81$, $PNK^L = .19$. With Yates correction: blacks, $\chi^2 = 0.095$, $P > .95$; whites, $\chi^2 = 0.44$, $P > .80$.

phenotype observed in the population sample and the numbers expected according to the Hardy-Weinberg equilibrium.

Family Studies

Of the 51 white donors examined only one was found to have PNK activity in the PNK^L range. The results of family studies of this white PNK^L subject and of a black family with enzyme activity in the PNK^L range are presented in figure 3. These pedigrees are consistent with the proposal that a single gene pair governs red cell PNK activity.

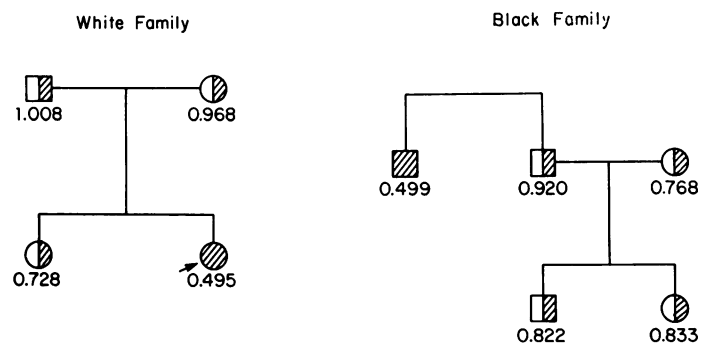

FIG. 3.—The partial pedigrees of a white and a black family. Numbers are PNK activity (mU/g Hb) in red cells.

Biochemical Studies

Thermostability. No difference could be detected in the thermal stabilities of erythrocyte PNK from a white and a black subject or the white PNK^L subject at temperatures ranging from 40°C to 65°C, at pH 6.85 and pH 7.58.

pH optimum. Similar pH-optimum curves were obtained in the range from pH 5.5 to 10.5 for erythrocyte PNK from white and black donors or the white PNK^L subject. A broad pH optimum at about 8.5 was present.

Stability at acidic pH. Stability studies of PNK stored at pH 5.84 at 4°C overnight showed the rate of inactivation was the same for the enzyme from white and black individuals or the white PNK^L subject.

Electrophoretic Studies

Starch gel electrophoretic patterns of PNK from white PNK^H and PNK^L subjects and a mixed sample of the two enzyme solutions with approximately the same protein content are shown in figure 4. No difference of electrophoretic mobil-

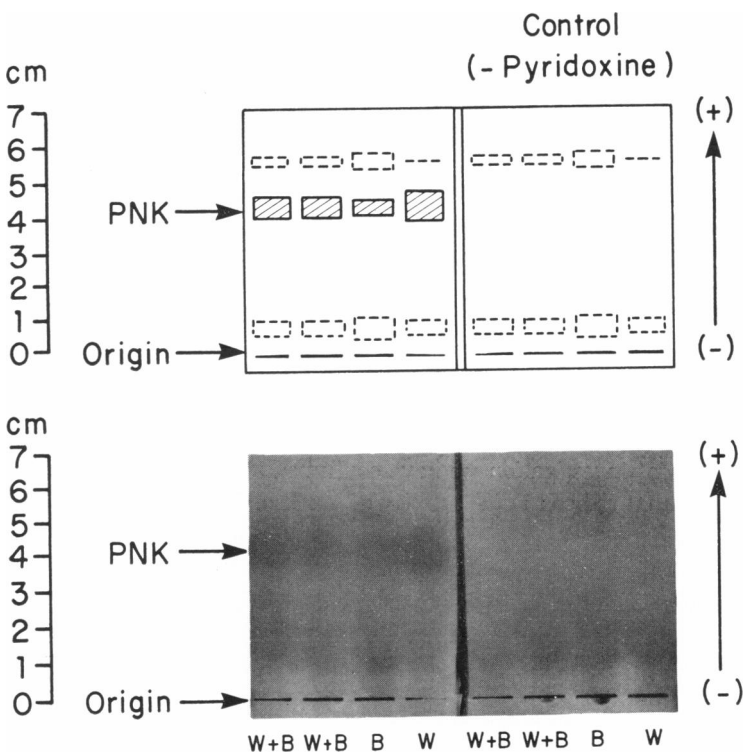

FIG. 4.—Starch gel electrophoresis of PNK in red cells of PNK^H (W) and PNK^L (B) subjects. W + B = mixture of samples. See text for detailed experimental conditions for electrophoresis. Duplicate gel (control) was stained with staining mixture for PNK activity in absence of pyridoxine. Activity band of pyridoxine kinase is PNK.

ity was detected. Electrophoresis in Tris-PO_4, Tris-citrate, or Tris-Cl buffer, pH 8.0, with the same concentrations used in the potassium phosphate, pH 7.0, system (see Materials and Methods) revealed no electrophoretically detectable structural differences between PNK from white PNK^H and black PNK^L individuals. Inclusion of 0.5 mM ATP and 0.5 mM $MgCl_2$ in the Tris-PO_4, pH 8.0 system did not affect the results. Under similar experimental conditions, it was also found that erythrocyte PNK^L from the white subject (40 μl of a dialyzed enzyme solution: 5.22 mU/ml) had the same electrophoretic mobility as that observed for enzymes from either the white or black subject.

PNK Activities in Young and Old Red Cells

Our previous investigations have shown that PNK from normal white individuals declines in activity as red cells age; after centrifugation the upper layer of red cells, enriched with young erythrocytes, has approximately 1.6 times the activity of the lower layer, enriched with older red cells [4]. The upper, young cell-enriched fractions from the white PNK^L subject and black PNK^L donors had approximately 2.2-fold more PNK activity than that in the old cell-enriched fraction. The enzyme activity in the younger red cells from the PNK^L was about 60% of that from PNK^H donors, but the activity of older red cells from PNK^L donors was only about 30% of that from PNK^H donors. Hexokinase was used as an independent marker for red cell age. No significant difference was observed between PNK^L and PNK^H subjects with respect to hexokinase activity either of the more or less dense red cell fraction. The rate of decline of enzyme activity with aging of red cells can be quantitated by plotting the activity of PNK in fractions of various density (age) against hexokinase. The mean rate of decline of enzyme activity with aging in white and black PNK^L subjects was found to be significantly greater than that in PNK^H subjects (table 2). These results indicate that the degree of enzyme deficiency in younger PNK^L red cells was less severe than in older red cells or the unfractionated red cells.

DISCUSSION

In the present investigation we have confirmed and extended our previous observation [1, 2] that red cell PNK activity of blacks is lower than that of whites. With

TABLE 2

RATE OF DECLINE OF PYRIDOXINE KINASE ACTIVITY IN RED CELLS UPON AGING

No. Subjects	Phenotype	Race	Slope of Regression of Log PNK on Log Hexokinase (mean ± SD)
7	PNK^H	W	0.53 ± 0.27
3	PNK^L	B	1.13 ± 0.08
1*	PNK^L	W	1.16 ± 0.27

* From three determinations on one subject. t(1 vs. 2) = 5.4, $P < .001$; t(1 vs. 3) = 3.4, $P < .01$; t(2 vs. 3) = 0.18 N.S.

the larger sample we have now examined, a probability plot provides a suggestion of trimodality of the enzyme distribution, making it possible to provisionally separate the subjects into three phenotypes, PNK^H, PNK^I, and PNK^L. We suggest that these represent three genotypes, PNK^H/PNK^H, PNK^H/PNK^L, and PNK^L/PNK^L. Employing the necessarily arbitrary dividing values provided by the population data, a remarkably good fit is obtained applying the Hardy-Weinberg equation to both the black and white population. The gene frequency of PNK^L is estimated at .65 in the black sample. Since the gene pool of the California black population is diluted to an extent estimated to be approximately 22% by genes of white origin [8], one may speculate that the frequency of the PNK^L gene may approach 1.0 in African populations. We hope to have an opportunity to examine such populations.

In order to detect a possible qualitative difference between the enzyme found in PNK^L and PNK^H individuals, we have investigated thermal stability, pH-activity curve, and inactivation at acidic pH of PNK from red cells of white and black subjects and the white PNK^L subject. These results and previously reported comparisons of biochemical properties, such as K_m for pyridoxine, chromatographic mobility on DEAE-cellulose, and susceptibility to inhibition by 4-deoxypyridoxine of the kinases from whites and blacks [4], all fail to demonstrate any significant differences between the enzymes from these two races. Comparison of the enzymes by isoelectric focusing could not be carried out because of the instability of the enzyme at the pI range [4]. However, we have also been able to design an activity staining procedure for PNK on starch gels by coupling to pyruvate kinase and lactic dehydrogenase. Since only 1.3 mU/g Hb of PNK is present in human red cells, direct starch gel electrophoresis of hemolysate provides insufficient activity for satisfactory visualization. This limits the screening of samples from a large population. However, using a partially purified enzyme prepared by DEAE-cellulose chromatography concentrated by ammonium sulfate precipitation, PNK activity can be visualized after electrophoresis. The same electrophoretic mobility on starch gels and similar biochemical properties of PNK from white and black donors and the white PNK^L failed to reveal a qualitative difference between the enzymes from these two races.

In spite of the normal thermal stability of PNK activity from deficient individuals, diminished red cell (not leukocyte or fibroblast) enzyme activity made the possibility of in vivo instability of the enzyme attractive. This discrepancy between leukocyte and erythrocyte enzyme may occur with stability mutants because of the relatively long life span of the nonnucleated erythrocyte. It is interesting, in this respect, that $G6PD^{A-}$, clearly a stability mutation [9], does not betray its in vivo lability in in vitro heat stability tests. Examination of age fractionated erythrocytes for PNK activity did reveal a more rapid rate of decline in individuals with low activity than those with high activity. Thus, it appears that the PNK^L state in red cells is probably the result of a stability mutation. We cannot, however, rule out the possibility that it results from other changes within the red cell which affect the stability of PNK.

Only a few biochemical differences have been observed between blood samples

from black and white donors. Black subjects appear to have lower red cell galactokinase activity [10] and higher serum creatine phosphokinase activity [11] than whites. A slight difference in ATP levels was also observed [12], and may be secondary to the slightly lower levels of serum phosphate in blacks [13]. Differences in sodium and potassium content of red cells have also been reported [14].

The marked difference in the frequency of the putative PNK^L gene in blacks and whites suggests that it may exert a selective advantage in the African environment. Two other important polymorphisms affecting primarily Africans, the gene for sickle cell hemoglobin and for $G6PD^{A-}$ have both been shown to confer resistance to falciparum malaria. Because the gene for low PNK activity exerts its effect only in erythrocytes suggests that it, too, may confer resistance to malaria. It is of interest in this respect, that most Africans enjoy complete immunity to infection with vivax malaria [15]. If this immunity is a function of a single gene, it must be one which is very prevalent in the African population. A PNK^L gene would fill this requirement. We hope to be able to carry out investigations which will test the effect of PNK deficiency on resistance to vivax malaria.

SUMMARY

The mean PNK activity in red blood cells from black subjects was only about 40% of that in whites. Among 51 whites examined, one was found to have enzyme deficiency. The estimated gene frequencies for PNK^H (the common allele in whites which codes for higher enzyme activity) and PNK^L (the common allele in blacks which codes for lower enzyme activity) were .35 and .65, respectively, for black donors, and .81 and .19, respectively, for white donors. The variant enzyme in persons with enzyme deficiency was associated with an increased rate of degradation in red cells during aging. No other biochemical or electrophoretic differences were detected.

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Symposium on Inborn Errors of Metabolism

The National Foundation–March of Dimes is sponsoring a symposium on inborn errors of metabolism to be held Wednesday, March 24, 1976, at the William Penn Hotel in Pittsburgh, Pennsylvania. Participants include Barton Childs, M.D.; R.J. Desnick, Ph.D., M.D.; Prof. Dr. Hans Galjaard; William J. Mellman, M.D.; Elizabeth F. Neufeld, Ph.D.; Leon E. Rosenberg, M.D.; Harvey L. Sharp, M.D. For information or registration forms, please contact Mr. Robert McFarland, Greater Pittsburgh Chapter, National Foundation–March of Dimes, 1310 Fulton Building, Pittsburgh, Pennsylvania 15222. Telephone (415) 391-3193.